Arch Toxicol (2012) 86:805–813
DOI 10.1007/s00204-012-0804-8

GENOTOXICITY AND CARCINOGENICITY

Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells

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Received: 14 September 2011/Accepted: 11 January 2012/Published online: 14 February 2012

Abstract Glyphosate (G) is the largest selling herbicide worldwide; the most common formulations (Roundup, R) contain polyoxyethyleneamine as main surfactant. Recent findings indicate that G exposure may cause DNA damage and cancer in humans. Aim of this investigation was to study the cytotoxic and genotoxic properties of G and R (UltraMax) in a buccal epithelial cell line (TR146), as workers are exposed via inhalation to the herbicide. R induced acute cytotoxic effects at concentrations [40 mg/l after 20 min, which were due to membrane damage and impairment of mitochondrial functions. With G, increased release of extracellular lactate dehydrogenase indicative for membrane damage was observed at doses [80 mg/l. Both G and R induced DNA migration in single-cell gel electrophoresis assays at doses [20 mg/l. Furthermore, an increase of nuclear aberrations that reflect DNA damage was observed. The frequencies of micronuclei and nuclear buds were elevated after 20-min exposure to 10–20 mg/l, while nucleoplasmatic bridges were only enhanced by R at the highest dose (20 mg/l). R was under all conditions more active than its active principle (G). Comparisons with results of earlier studies with lymphocytes and cells from internal organs indicate that epithelial cells are more susceptible to the cytotoxic and DNA-damaging properties of the herbicide and its formulation. Since we found genotoxic effects after short exposure to concentrations that correspond to a 450-fold dilution of spraying used in agriculture, our findings indicate that inhalation may cause DNA damage in exposed individuals.

Keywords Glyphosate ffRoundup ffCytotoxic ffi Comet assay ffMicronuclei ffBuccal cells

Abbreviations

BN-MNi	Binucleated cells with micronuclei
G	Glyphosate
LDHe	Extracellular lactate dehydrogenase
MNi	Micronuclei
NB	Nuclear buds
NDI	Nuclear division index
NPB	Nucleoplasmic bridge
NR	Neutral red
R	Roundup-Ultramax
SRB	Sulforhodamine B
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide

Introduction

Glyphosate (G) is one of the most widely used herbicides worldwide; in 2007, its annual global use exceeded 907,000 tons (REUTERS 2011).

Since its solubility and ionic nature retard the penetration into plant tissues, the native compound is mixed with surfactants; one of the most widely used is polyethoxylated tallow amine (POEA), the common trade name of these formulations being Roundup (R) (Williams et al. 2000).

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The health effects of G and R have been studied intensely since these products entered the market in the 1970s (for reviews see Williams et al. 2000), and it was concluded that they can be regarded as safe for humans (Agency 1993; WHO 1994; Williams et al. 2000). The assumption that the herbicide and its preservations do not cause DNA damage and cancer in humans is based on the results of long-term carcinogenicity studies with rodents in which the herbicide was fed or injected i.p. to the animals and also on findings from genotoxicity studies (for details see ''Discussion'' section).

However, in the last decade, a number of studies have been published, which indicate that occupational exposure of humans to the pesticide is associated with increased cancer risks (Bolognesi et al. 2009; De Roos et al. 2005, 2003; Eriksson et al. 2008; Hardell and Eriksson 1999; Hardell et al. 2002; McDuffie et al. 2001). Furthermore, it was found in field studies that contact of agricultural workers due to spraying leads to induction of micronuclei (MNi) that are formed as a consequence of structural and numerical chromosomal aberrations and to comet formation in peripheral lymphocytes (Bolognesi et al. 2009; Paz-y-Miño et al. 2007). Also in the course of the production of the pesticide, humans may come into dermal and/or inhalative contact with the herbicide, but no data are available from biomonitoring studies in which DNA damage was measured in exposed factory workers.

In order to draw conclusions whether effects may occur due to occupational exposure, additional in vitro studies are required in which dose response effects and the molecular mechanisms are investigated. At present, some results are available, which have been obtained in experiments with lymphocytes and with human cell lines derived from different internal organs. The outcomes of these investigations are quite controversial (Benachour and Seralini 2009; De Roos et al. 2005; Gehin et al. 2005; Lueken et al. 2004; Mladinic et al. 2009a; Monroy et al. 2005).

Primary aim of the present study was to find out whether exposure to G and R causes adverse effects in the human-derived buccal epithelial cell line TR146 (Jacobsen et al. 1999). In cytotoxicity experiments, four different endpoints were used, which reflect different modes of action. The release of lactate dehydrogenase (LHDe) was monitored to provide information about damage of the cell membranes (Maines 1998); alterations of mitochondrial functions were assessed by determination of the activity of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (Brosin et al. 1997). Furthermore, changes of cell proliferation were determined by use of sulforhodamine B (SRB) that binds electrostatically to proteins and correlates therefore with the total protein content of the cells (Skehan et al. 1990). Additionally, experiments with neutral red were conducted to study changes of the integrity of the membranes and of lysosomal activity (Repetto et al. 2008). To find out whether G and R affect the DNA stability of the cells, single-cell gel electrophoresis assays (SCGE) were conducted under standard alkaline conditions that reflect the formation of single- and double-strand breaks and apurinic sites. These experiments are based on the measurement of DNA migration in an electric field (Tice et al. 2000). Additionally, we conducted cytokinesis-block MN cytome assays with the TR146 cell line according to the protocol of Fenech (2007) in which different nuclear anomalies were measured. Some of them are indicative for DNA alterations namely MNi in binucleated (BN) cells that are formed as a consequence of structural and numerical chromosomal aberrations (Fenech 2007), nuclear buds (NB) that contain amplified DNA expelled from the main nucleus, whole chromosomes, or fragments (Dutra et al. 2010), and nucleoplasmic bridges (NBP) that reflect formation of dicentric chromosomes (Fenech 2007). Additionally, different forms of cell death, namely apoptosis and necrosis, were registered.

The experiments were conducted in such a way that they reflect exposures that may occur under occupational conditions, i.e., the cells were treated for short time periods with dilutions of R and G that are used in spraying solutions.

Materials and methods

Storage and cultivation of the indicator cells

The human cell line TR146, which is derived from a human neck metastasis of buccal epithelial origin (Rupniak et al. 1985), was cultured under standard conditions (37ffC moist atmosphere of 5% CO_2) in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma Aldrich Chemical Co., St. Louis, MO). The medium was changed every 2–3 days; when the cultures reached confluency, the cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, Lonza, Verviers, Belgium), detached with TrypLE Express (Gibco, Invitrogen, Carlsbad, CA, USA), centrifuged, and subcultured. TR146 cells express ultrastructural characteristics of normal human buccal epithelial cells, e.g., intermediate filaments, microvilli-like processes, and lack of complete keratinization (Jacobsen et al. 1999).

Chemicals

Glyphosate (purity 95% powder; Monsanto Europe S.A) and Roundup (Roundup Ultra Max, 450 g/l glyphosate

acid, Monsanto Europe S.A) were dissolved and diluted immediately before use in DMEM.

Acute toxicity assays

For cytotoxicity experiments, 5 9 10^4 indicator cells were seeded in 96-well plates and exposed to different concentrations of G and R solutions for 20 min. These experiments allow to draw conclusions on the impact of the herbicide on the damage of the cells due to different mechanisms. Toxicity due to damage of the cell membranes was determined with the extracellular LDHe assay that is based on the measurement of the oxidation of NADH to NAD^+ (Maines 1998). Alterations of mitochondrial functions were studied in XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide) assays that measure succinate dehydrogenase activity of viable cells (Brosin et al. 1997). The SRB test was used to monitor total protein synthesis as a marker of cell proliferation (Vichai and Kirtikara 2006). SRB binds to cellular proteins and can be quantified after solubilization. All three assays were conducted with commercially available test kits (Xenometrix AG, Allschwil, Switzerland). Furthermore, neutral red assays were conducted according to the protocol of Repetto et al. (2008) in which uptake of the dye by healthy cells was monitored as an endpoint. These experiments provide information about membrane integrity and lysosomal activities of the cells. All experiments are based on spectrophotometric measurements and were evaluated with an automated microplate reader (Tecan Infinite 200 PRO). In all experiments, three measurements were performed per dose and repeated at least once.

Single-cell gel electrophoresis (SCGE) assays

SCGE assays were performed according to the guidelines described by Tice et al. (2000).

In order to determine the impact of the pure compound and of the formulation on the DNA stability, 2 9 10^5 TR146 cells were seeded in 24-well plates and allowed to attach (37ffC; 5% CO_2). Thereafter, the culture medium was replaced by 400 ll of different concentrations of the herbicide, which had been dissolved in serum-free medium. After incubation in the dark (37ffC; 5% CO_2) for 20 min, the medium was discarded and the cells washed twice with DPBS and detached with TrypLE Express. After two washing steps with DMEM and centrifugation (200xg, 8 min, 21ffC), the pellets were resuspended in low melting agarose (LMA, 0.5%). Then, they were spread onto precoated agarose slides (1.5% normal melting agarose) and lysed in the dark at 4ffC for at least 1 h. After 20 min unwinding in electrophoresis solution (pH C 13), electrophoresis was carried out for 20 min (300 mA, 25 V) and neutralization was performed twice for 8 min. Air dried slides were stained with ethidium bromide (20 lg/ml), and the percentage of DNA in tail was measured by use of a computer-aided image analysis system (Comet IV, Perceptive Instruments Ltd., Haverhill, UK).

For each experimental point, three cultures were made in parallel; from each culture, one slide was prepared, and from each, 50 randomly distributed cells were evaluated.

Cytokinesis-block micronucleus (CBMN) cytome assay

The CBMN assay was performed by use of the cytochalasin B technique described by Fenech (2007).

Into 6-well plates, 4.5 9 10^5 cells were seeded and were allowed to attach overnight. After treatment for 20 min with the test compounds or 100 lg/ml methyl methanesulfonate (MMS, positive control; Sigma Aldrich, St. Louis, MO, USA), the cells were washed twice with PBS and cultured further in DMEM containing 10% FCS and cytochalasin B (final concentration, 3.0 lg/ml; Sigma Aldrich, St. Louis, MO, USA). The total incubation time of the cultures was 48 h. Subsequently, the cells were washed twice with DPBS and were harvested by trypsinization; slides were made by the cytocentrifugation method (Fenech 2007) and were air dried, fixed, and stained with Diff Quick (Dade Behring, Deerfield, IL, USA).

The total number of micronuclei in binucleated cells (MNi) as well as the number of binucleated cells with micronuclei (BN-MNi), NB, NPB, and apoptotic and necrotic cells was determined according to the criteria defined by Fenech (2007). Cells that divided after addition of cytochalasin B can be recognized as binucleated. The restriction of microscopic analysis to MNi in BN cells does not enable the detection of confounding effects caused by suboptimal or altered cell division kinetics; therefore, the nuclear division index (NDI) was determined in 500 cells (Fenech 2007). For each experimental point, TR146 cultures were prepared in triplicate. From each culture, [1,000 binucleated cells were evaluated under 400-fold magnification (Nikon Photophot-FXA, Tokyo, Japan).

Statistical analysis

Data analyses of the cytotoxicity and SCGE assays were performed with the GraphPad Prism 5 Project software system (La Jolla, CA, USA). Results are reported as means ± standard deviations (SD). The results were analyzed using one-way ANOVA and Dunnett's test; p values B0.05 were considered as statistically significant.

The chi-square test with Yate's correction was used for the evaluation of the CBMN experiments; p values B0.05 were considered statistically significant.

Results

TR146 cells have been used earlier in cytotoxicity experiments (Boyle et al. 2010; Eirheim et al. 2004) but not to investigate the genotoxic effects of chemicals. The cells have, in nonconfluent cultures, an oval shape and possess large nuclei, their morphology resembling that of differentiated epithelial cells from the buccal cavity. They possess specific ultrastructural characteristics of normal human buccal epithelium, e.g., intermediate filaments, microvilli-like processes, and absence of complete keratinization (Jacobsen et al. 1999).

Prior to the main experiments, we determined in several experimental series a number of standard parameters. In SCGE assays, the average of % DNA in tail (mean ± SD) of untreated cells was 8.89 ± 1.95 (values are results obtained in 4 independent experiments with 3 cultures per experimental point). After addition of cytochalasin B (3.0 lg/ml) for 48 h, [75% of the cells were binucleated and the NDI in untreated cultures was 1.89 ± 0.09. BN cells with MNi and the total number of MNi, NB and NPB could be easily quantified; their frequencies in untreated cultures were 4.12 ± 1.41, 4.48 ± 2.02, 3.90 ± 2.74, and 4.88 ± 3.16, respectively. Treatment of the cells with 100 lg/ml of the alkylating agent MMS for 20 min caused significant induction of BN cells with MNi (9.74 ± 0.56) and total number of MNi (10.19 ± 0.73), as well as NB (8.37 ± 1.34) and NPB (9.92 ± 2.07). All values are means of results obtained with three independent cultures.

The results of experiments in which the impact of G and R on the different cellular integrity parameters was studied are shown in Fig. 1a–d. It can be seen that clear differences between the effects of pure G and its formulation were found; the latter was in all cases more toxic than the herbicide. The most sensitive assays were the LDHe test and the XXT (Fig. 1a, b). In the last, a significant effect of R was seen at a dose level [40 mg/l while a clear increase of the LDHe levels was seen already with 10 mg/l. The SRB and the NR assays were less responsive; significant alterations were seen at 80 and 100 mg/l, respectively (Fig. 1c, d). All effects increased as a function of the exposure concentrations; in the case of LDHe, they reached a maximal value at 40 mg/l. The LC_{50} values were assessed on the basis of the experimental data, the lowest value being found in the XXT test (* 100 mg/l) followed by the NR assay (140 mg/l) and the SRB (150 mg/l) assay. In contrast

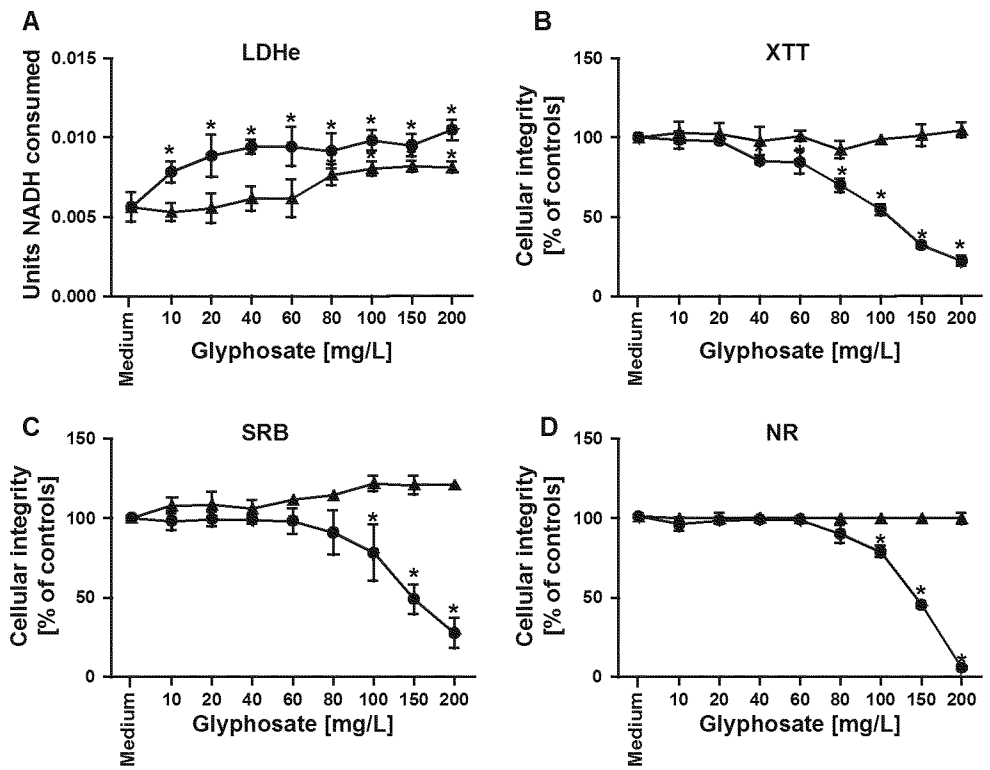

Fig. 1 a–d Impact of G and R on cellular integrity parameters in the human buccal epithelial cell line TR146. The cells were treated with different concentrations of the herbicide (triangle with cross line) G and its formulation (circle with cross line) R for 20 min. Subsequently, alterations of the different endpoints were monitored spectrophotometrically as described in ''Materials and methods''. Each experimental point represents the means ± SD of three measurements per experimental point. Asterisks indicate significant differences from control values (Dunnett's test, p B 0.05)

to R, no significant effects were seen in these experiments with G, except in the LDHe assay; a clear increase of the enzyme activity was seen in this test with doses [80 mg/l.

Figure 2a, b depicts the results of the SCGE assays that were conducted under standard conditions that reflect strand breaks and formation of apurinic sites. G and R induced in these experiments a significant dose-dependent increase of DNA-migration. The x-axis of Fig. 2b indicates the glyphosate concentrations that were contained in the R formulation. The LOELS for G and R were in the SCGE assays 20 mg/l. The most pronounced effects were seen with R at the highest dose levels tested (150 and 200 mg/l), but under these conditions, a pronounced decrease of the cellular integrity was seen in the NR assay. Therefore, it can be not excluded that cytotoxic effects caused by high concentrations may have led to misleading results in the SCGE assays (Hartmann et al. 2003). However, significant effects were also observed in the present study with lower exposure concentrations that did not affect the cellular integrity of the cells.

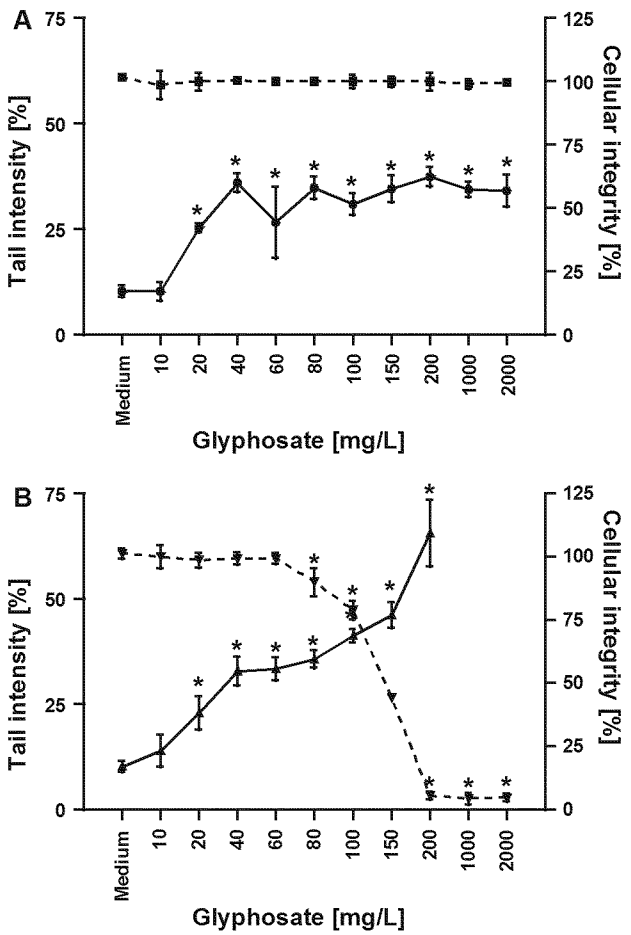

Fig. 2 a, b Impact of G (A) and R (B) on the cellular integrity and on DNA migration in the human-derived epithelial buccal cell line TR146. The cells were treated with different concentrations of the test compounds for 20 min. Subsequently, DNA damage was analyzed, and the cellular integrity was determined as described in ''Materials and methods''. Each experimental point represents results (means ± SD) obtained with three parallel cultures. From each, 50 cells were evaluated for comet formation (solid line). The cellular integrity (dotted line) was determined with Neutral Red (data represent means of three experiments in which the means of the cellular integrity of three cultures were determined). Asterisks indicate significant differences from control values (Dunnett's test, p B 0.05)

The findings obtained in the CBMN assays with the herbicide and its formulation are summarized in Fig. 3a–f. The results obtained with G are represented by the black bars; the results obtained under identical experimental conditions with the formulation are depicted as shaded bars. Exposure of the cells for 20 min led to a significant and dose-dependent increase of MNed cells and of the total number of MNi (Fig. 3a, b). Also the numbers of NB (Fig. 3c) were increased as a function of the exposure concentrations (p \ 0.0001). In the case of the NPB (Fig. 3d), the only significant effect was obtained with the highest dose of R (20 mg/l); while with G, no positive result was obtained under all experimental conditions. The NDI values were not altered after treatment of the cells with the chemicals (data not shown). The numbers of necrotic cells were increased in cells exposed to highest concentrations of R and G (p \ 0.006 in all cases, Fig. 3e). The number of apoptotic cells was significantly increased at the highest concentration (20 mg/l) of G (p \ 0.01, Fig. 3f).

Discussion

Our results show that R, but not its active principle G, causes pronounced cytotoxic effects in human-derived buccal epithelial cells. Furthermore, the genotoxicity tests show that the herbicide as well as its formulation induces strand breaks that lead to formation of comets as well as nuclear anomalies that reflect DNA instability including chromosomal damage.

In general, similar effects were observed with the four cytotoxicity assays under identical experimental conditions, despite the fact that they reflect different mechanisms of cell damage. As described above, G did not induce effects in the NR, SRB, and in the XXT assay up to concentrations of 200 mg/l, while a clear effect was seen in the LHDe assay with doses C80 mg/l. On the contrary, significant cytotoxic effects were observed with R in all four assays, and significant changes were seen in the LDHe assay and in the XTT test already at doses [10 mg/l indicating that the formulation damages the cell membranes and interferes with protein synthesis at these low concentrations.

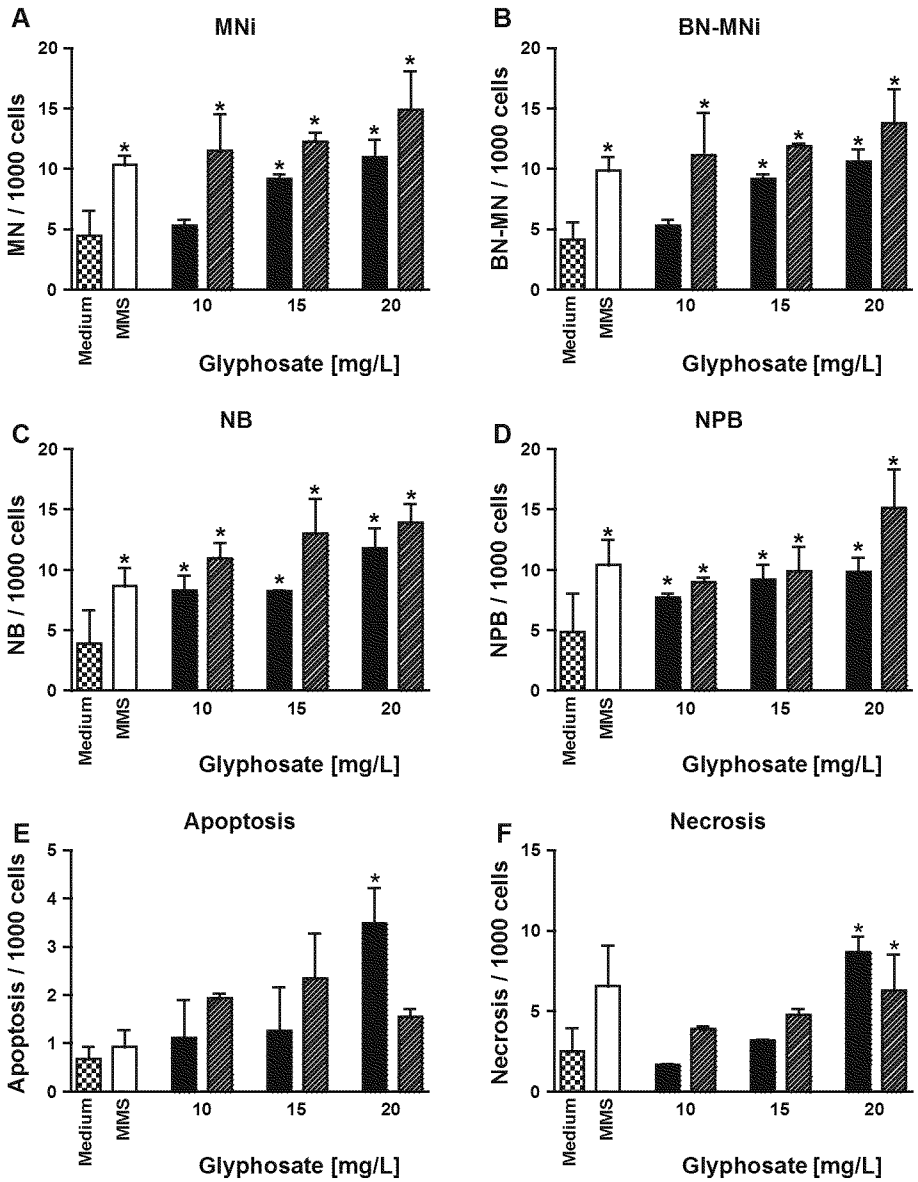

Fig. 3 a–f Impact of G and R on the frequencies of MNi, BN-MNi, NB, NPB and on apoptosis as well as necrosis in human-derived buccal epithelial cells (TR146). The cells were treated with aqueous solutions of G and R for 20 min. Subsequently, they were washed twice with DPBS, and cytochalasin B (3.0 lg/ml) was added for 48 h. The different endpoints were determined as described by Fenech (2007). Bars represent the means ± SD of results obtained with three cultures; from each, [1,000 cells were evaluated. Black bars show findings obtained with pure G; shaded bars represent results obtained with R (the concentrations were established in such a way that the G levels in the formulation corresponded to those of the pure active component G). MMS (100 lg/ml; 20 min) was used as a positive control. Asterisks indicate statistical significance from negative control values (chi-square test with Yate's correction, p B 0.001)

The cytotoxic properties of different R preparations and of G have been studied earlier in different human-derived cell lines. The findings show in agreement with ours that R formulations are in general more cytotoxic than G (Benachour and Seralini 2009; Benachour et al. 2007; Gasnier et al. 2009; Monroy et al. 2005; Richard et al. 2005). Comparisons of the sensitivity of different toxicity tests indicate that R causes membrane damage at lower concentrations than inhibition of mitochondrial functions in embryonic (293) and placental-derived cells (JEG3), while umbilical vein cord endothelial cells (HUVEC) were equally sensitive to both endpoints (Benachour and Seralini 2009). High concentrations of G affected in these experiments primarily mitochondrial functions (which were detected with the MTT assay) while no effects were seen in an assay that detects changes of cell membrane functions (by release of cytoplasmic adenylate kinase) (Benachour and Seralini 2009). This latter observation is in contrast to the present findings, but it should be noted that other test systems and longer exposure periods (24 h) were used. G was tested additionally in several other human lines that originated from different organs including fibrosarcoma and hepatoma cells as well as keratinocytes, and in all of them, weak effects were only seen at high dose levels (Gasnier et al. 2009; Gehin et al. 2005; Monroy et al. 2005).

The results of the SCGE experiments show that G as well as R induces comet formation under standard (alkaline) conditions that reflect strand breaks and apurinic sites (Tice et al. 2000). It can be seen in Fig. 2a, b that the

effects increased as a function of the exposure concentrations and that DNA migration was also observed under conditions that did not affect the cellular integrity. Also in earlier investigations with human-derived cells, SCGE assays were used to monitor the DNA-damaging effects of the herbicide. Significant induction of DNA migration was seen in human hepatoma cells (HepG2) with a specific formulation (Roundup 400) with G levels [5 mg/l after 24-h treatment (Gasnier et al. 2009). Other cell lines such as normal human fibroblasts (GM38) and the human fibrosarcoma line HT1080 were less sensitive in long-term (72 h) exposure studies, and clear positive effects were seen only at concentrations [650 and 900 mg/l, respectively (Monroy et al. 2005). Mladinic et al. (2009a, b) studied the effects of G in human lymphocytes, and in absence of liver homogenate (S9), a significant effect was restricted to high concentrations ([580 mg/l). However, after addition of liver homogenate (S9 mix), comet formation was observed at much lower dose levels (i.e., at 3.5 mg/l). Taken together, these effects show that distinct differences exist in the sensitivity of cells from different organs and indicate that drug-metabolizing enzymes, which are represented in the enzyme activation mix and in HepG2 cells, increase the DNA-damaging properties of the herbicide.

Also in different in vivo experiments, positive results were obtained in SCGE experiments, for example in amphibians (Clements et al. 1997), reptiles (Poletta et al. 2009), fish (Cavas and Konen 2007; Guilherme et al. 2010), and plants (Alvarez-Moya et al. 2011). Few years ago, the first article was published by Paz-y-Miño et al. (2007) in which a significant increase of comet formation in peripheral lymphocytes of humans exposed to G via spraying was reported.

It has been stressed by Williams et al. (2000) that the positive results that were obtained in older SCGE experiments may be due to secondary effects, for example to cytotoxicity that can cause misleading results (Hartmann et al. 2003). This confounding factor can be excluded in the present investigation.

The most relevant finding of the present study is the observation of a significant, dose-dependent induction of two types of nuclear anomalies that reflect genomic damage by G and its formulation. As shown in Fig. 3a–c, the former markers (MNi, BN-MNi, and NB) were significantly increased after exposure of the cells. The most sensitive endpoint was MNi induction; treatment of the cells with highest dose of R (20 mg/l) caused a threefold increase over the background, and with the corresponding concentration of G, a weaker effect was seen (Fig. 3a, b). NPB was a less responsive endpoint, and the only significant effect was obtained with the highest R dose (20 mg/l).

These results indicate that the damage seen in the SCGE assays is not completely repaired but leads to persisting alterations of the genetic material. The different endpoints that we analyzed are caused by different molecular mechanisms. MNi reflect numerical as well as structural chromosomal aberrations, while NB are formed as a consequence of gene amplification or expulsion of intact chromosomes or fragments; NPB are caused by formation of dicentric chromosomes (Fenech 2007).

Mladinic et al. (2009a) compared the effects of G and several other herbicides in cytome experiments with human lymphocytes with and without addition of exogenous activation mix (liver homogenate, S9). They found at a high concentration (580 mg/l) without S9 a significant increase of NB but not of MNi and NPB after exposure for 4 h. Addition of enzyme homogenate led to an increase of the MNi frequencies at concentrations that reflect conditions reached as a consequence of occupational exposure. In this context, it is also notable that other endpoints (NB and NPB) were elevated under these conditions but only at high concentrations. Earlier investigations with human and bovine lymphocytes in which chromosomal aberrations were monitored without enzyme homogenate yielded positive results after G treatment at low doses (1.4 mg and 2.9 mg/l) (Lioi et al. 1998a, b) while in another study with human lymphocytes, consistently negative results were obtained with higher concentrations (Williams et al. 2000).

G and R preparations were also tested in MNi assays with rodents, fishes, and reptiles. The findings obtained with mice are controversial. In most investigations (i.e., in eight out of ten), negative results were obtained in polychromatic erythrocytes from the bone marrow (for details see Dimitrov et al. 2006; Grisolia 2002; Williams et al. 2000) while positive results were reported in erythrocytes of mice (Bolognesi et al. 1997), fishes (Cavas and Konen 2007; Grisolia 2002), and caimans (Poletta et al. 2009). The MNi assay with polychromatic erythrocytes from the bone marrow is at present the most widely used procedure for routine testing of chemicals, but it has the disadvantage that reactive and short-lived metabolites may not reach the target cells. Thus, false negative results were obtained in bone marrow MN studies with representatives of certain classes of potent DNA-reactive carcinogens such as heterocyclic aromatic amines and nitrosamines (Hayashi et al. 1989). Therefore, the lack of a positive result with G and R in these experiments does not prove that the test compounds are safe in regard to their genotoxic properties. So far, only one biomonitoring study with humans is available in which MNi frequencies were monitored in G exposed workers in Colombia, and significantly higher frequencies were found than in unexposed individuals (Bolognesi et al. 2009). Taken together, the currently available data show that the effects of the herbicide and its formulation depend

strongly on the experimental system and on the type of indicator cells used.

The findings of the present study suggest that buccal epithelial cells are more sensitive toward the cytotoxic and DNA-damaging effects of G and R than cells from the hematopoietic system. The R formulation that we tested contains 450 g/l of G and should be diluted according to the instructions of the manufacturer to 1–3% before use (final concentration 4,500–13,500 mg/l). The fact that we found significant acute and genotoxic effects at levels between 10 and 20 mg/l after 20 min indicates that short contact with a 225–1,350-fold dilution of the spraying solution may cause adverse effects in cells from the oral cavity (and possibly also in other respiratory epithelia). In this context, it is notable that the experiments of Benachour et al. (2007) showed that the cytotoxic effects of G and several formulations increase strongly as a function of the exposure time, and further experiments are under way to study the dose and time kinetics of the herbicide in more detail in epithelial cells.

It has been shown by Bonassi et al. (2011) that increased MNi frequencies in lymphocytes are valid biomarkers for cancer risks in humans, and it is notable that a number of studies have been published in the last years, which indicate that correlations exist between exposure to G and elevated cancer incidences in humans (De Roos et al. 2005, 2003; Eriksson et al. 2008; Hardell and Eriksson 1999; Hardell et al. 2002; McDuffie et al. 2001). Our findings support the assumption of a possible association between G exposure and increased cancer risks and underline the importance of further human studies for example of MNi experiments with exfoliated buccal cells from agricultural workers and laborers that are exposed in factories. At present, no such data are available and estimates of inhalative exposure due to spraying, which are mentioned in the review of Williams et al. (2000), are vague and have not been published in scientific journals. It is known that more than 90% of all human cancers are of epithelial origin (Cairns 1975). Therefore, the present observation of induction of DNA damage under conditions that are relevant for humans in epithelial cells derived from the oral cavity should be takes as an indication for potential adverse effects.

References

Alvarez-Moya C, Silva MR, Arambula AR, Sandoval AI, Vasquez HC, Gonzalez Montes RM (2011) Evaluation of genetic damage induced by glyphosate isopropylamine salt using Tradescantia bioassays. Genet Mol Biol 34:127–130

Benachour N, Seralini GE (2009) Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem Res Toxicol 22:97–105

Benachour N, Sipahutar H, Moslemi S, Gasnier C, Travert C, Seralini GE (2007) Time- and dose-dependent effects of roundup on human embryonic and placental cells. Arch Environ Contam Toxicol 53:126–133

Bolognesi C, Bonatti S, Degan P, Gallerani E, Peluso M, Rabboni R, Roggieri P, Abbondandolo A (1997) Genotoxic activity of glyphosate and its technical formulation roundup. J Agric Food Chem 45:1957–1962

Bolognesi C, Carrasquilla G, Volpi S, Solomon KR, Marshall EJ (2009) Biomonitoring of genotoxic risk in agricultural workers from five colombian regions: association to occupational exposure to glyphosate. J Toxicol Environ Health A 72:986–997

Bonassi S, El-Zein R, Bolognesi C, Fenech M (2011) Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26:93–100

Boyle MA, O'Donnell MJ, Russell RJ, Coleman DC (2010) Lack of cytotoxicity by Trustwater Ecasol used to maintain good quality dental unit waterline output water in keratinocyte monolayer and reconstituted human oral epithelial tissue models. J Dent 38:930–940

Brosin A, Wolf V, Mattheus A, Heise H (1997) Use of XTT-assay to assess the cytotoxicity of different surfactants and metal salts in human keratinocytes (HaCaT). A feasible method for in vitro testing of skin irritants. Acta Derm Venereol 77:26–28

Cairns J (1975) Mutation selection and the natural history of cancer. Nature 255:197–200

Cavas T, Konen S (2007) Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (Carassius auratus) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis 22:263–268

Clements C, Ralph S, Petras M (1997) Genotoxicity of select herbicides in Rana catesbeiana tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. Environ Mol Mutagen 29:277–288

De Roos AJ, Zahm SH, Cantor KP, Weisenburger DD, Holmes FF, Burmeister LF, Blair A (2003) Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men. Occup Environ Med 60:E11

De Roos AJ, Blair A, Rusiecki JA, Hoppin JA, Svec M, Dosemeci M, Sandler DP, Alavanja MC (2005) Cancer incidence among glyphosate-exposed pesticide applicators in the agricultural health study. Environ Health Perspect 113:49–54

Dimitrov BD, Gadeva PG, Benova DK, Bineva MV (2006) Comparative genotoxicity of the herbicides Roundup, Stomp and Reglone in plant and mammalian test systems. Mutagenesis 21:375–382

Dutra A, Pak E, Wincovitch S, John K, Poirier MC, Olivero OA (2010) Nuclear bud formation: a novel manifestation of Zidovudine genotoxicity. Cytogenet Genome Res 128:105–110

Eirheim HU, Bundgaard C, Nielsen HM (2004) Evaluation of different toxicity assays applied to proliferating cells and to stratified epithelium in relation to permeability enhancement with glycocholate. Toxicol In Vitro 18:649–657

Eriksson M, Hardell L, Carlberg M, Akerman M (2008) Pesticide exposure as risk factor for non-Hodgkin lymphoma including histopathological subgroup analysis. Int J Cancer 123:1657–1663

Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc 2:1084–1104

Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Seralini GE (2009) Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 262:184–191

Gehin A, Guillaume YC, Millet J, Guyon C, Nicod L (2005) Vitamins C and E reverse effect of herbicide-induced toxicity on human epidermal cells HaCaT: a biochemometric approach. Int J Pharm 288:219–226

Grisolia CK (2002) A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides. Mutat Res 518:145–150

Guilherme S, Gaivao I, Santos MA, Pacheco M (2010) European eel (Anguilla anguilla) genotoxic and pro-oxidant responses following short-term exposure to Roundup–a glyphosate-based herbicide. Mutagenesis 25:523–530

Hardell L, Eriksson M (1999) A case-control study of non-Hodgkin lymphoma and exposure to pesticides. Cancer 85:1353–1360

Hardell L, Eriksson M, Nordstrom M (2002) Exposure to pesticides as risk factor for non-Hodgkin's lymphoma and hairy cell leukemia: pooled analysis of two Swedish case-control studies. Leuk Lymphoma 43:1043–1049

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR (2003) Recommendations for conducting the in vivo alkaline Comet assay. 4th international Comet assay workshop. Mutagenesis 18:45–51

Hayashi M, Sutou S, Shimada H, Sato S, Sasaki YF, Wakata A (1989) Difference between intraperitoneal and oral gavage application in the micronucleus test. The 3rd collaborative study by CSGMT/JEMS.MMS. Collaborative study group for the micronucleus test/mammalian mutagenesis study group of the environmental mutagen society of Japan. Mutat Res 223:329–344

Jacobsen J, Nielsen EB, Brondum-Nielsen K, Christensen ME, Olin HB, Tommerup N, Rassing MR (1999) Filter-grown TR146 cells as an in vitro model of human buccal epithelial permeability. Eur J Oral Sci 107:138–146

Lioi MB, Scarfi MR, Santoro A, Barbieri R, Zeni O, Di Berardino D, Ursini MV (1998a) Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures in vitro. Mutat Res 403:13–20

Lioi MB, Scarfi MR, Santoro A, Barbieri R, Zeni O, Salvemini F, Di Berardino D, Ursini MV (1998b) Cytogenetic damage and induction of pro-oxidant state in human lymphocytes exposed in vitro to gliphosate, vinclozolin, atrazine, and DPX-E9636. Environ Mol Mutagen 32:39–46

Lueken A, Juhl-Strauss U, Krieger G, Witte I (2004) Synergistic DNA damage by oxidative stress (induced by H2O2) and nongenotoxic environmental chemicals in human fibroblasts. Toxicol Lett 147:35–43

Maines MD (1998) In vitro methods for detecting cytotoxicity in current protocols in toxicology. Wiley, Hoboken

McDuffie HH, Pahwa P, McLaughlin JR, Spinelli JJ, Fincham S, Dosman JA, Robson D, Skinnider LF, Choi NW (2001) Non-Hodgkin's lymphoma and specific pesticide exposures in men: cross-Canada study of pesticides and health. Cancer Epidemiol Biomarkers Prev 10:1155–1163

Mladinic M, Berend S, Vrdoljak AL, Kopjar N, Radic B, Zeljezic D (2009a) Evaluation of genome damage and its relation to oxidative stress induced by glyphosate in human lymphocytes in vitro. Environ Mol Mutagen 50:800–807

Mladinic M, Perkovic P, Zeljezic D (2009b) Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay. Toxicol Lett 189:130–137

Monroy CM, Cortes AC, Sicard DM, de Restrepo HG (2005) Cytotoxicity and genotoxicity of human cells exposed in vitro to glyphosate. Biomedica 25:335–345

Paz-y-Miño C, Sánchez ME, Arévalo M, Muñoz MJ, Witte T, De-la-Carrera GO, Leone PE (2007) Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate. Genet Mol Biol 30:456–460

Poletta GL, Larriera A, Kleinsorge E, Mudry MD (2009) Genotoxicity of the herbicide formulation Roundup (glyphosate) in broad-snouted caiman (Caiman latirostris) evidenced by the Comet assay and the micronucleus test. Mutat Res 672:95–102

Repetto G, del Peso A, Zurita JL (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 3:1125–1131

REUTERS (2011) Roundup: cancer cause or crucial for food production? ''The Huffington Post''. http://www.huffingtonpost.com/2011/04/11/round-up-cancer-cause_n_847423.html

Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE (2005) Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ Health Perspect 113:716–720

Rupniak HT, Rowlatt C, Lane EB, Steele JG, Trejdosiewicz LK, Laskiewicz B, Povey S, Hill BT (1985) Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. J Natl Cancer Inst 75:621–635

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82:1107–1112

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221

USEP Agency (1993) Re-registration Eligibility Decision (RED): Glyphosate. US Environmental Protection Agency, Washington, DC

Vichai V, Kirtikara K (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 1:1112–1116

WHO (1994) Glyphosate. Environmental health criteria no. 159. World Health Organization, Geneva

Williams GM, Kroes R, Munro IC (2000) Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul Toxicol Pharmacol 31:117–165